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T Cell Cross-reactivity between Coxsackievirus and Glutamate Decarboxylase Is Associated with a Murine Diabetes Susceptibility Allele

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Summary

Limited regions of amino acid sequence similarity frequently occur between microbial antigens and host proteins. It has been widely anticipated that during infection such sequence similarities could induce cross-reactive T cell responses, thereby initiating T cell-mediated autoimmune disease. However, the nature of major histocompatibility complex (MHC)-restricted antigen presentation confers a number of constraints that should make this type of T cell cross-reactivity a rare, MHC allele-dependent event. We tested this prediction using two insulin-dependent diabetes mellitus (IDDM)-associated antigens, coxsackievirus P2-C (Cox P2-C) protein and glutamate decarboxylase (GAD65), which share a prototypic sequence similarity of six consecutive amino acids within otherwise unrelated proteins. We surveyed a panel of 10 murine MHC class II alleles that encompass the spectrum of standard alleles for the ability to cross-reactively present Cox P2-C and GAD65. Out of the 10 restriction elements tested, the sequence similarity regions were both dominant determinants and were cross-reactively displayed after the natural processing of whole antigens, only in the context of I-A^{mod}. These data show that cross-reactive T cell recognition of sequence similarity regions in unrelated proteins is confined to certain MHC alleles, which may explain MHC association with autoimmune disease. It is striking that these two diabetes-associated antigens were cross-reactively recognized only in the context of a diabetes susceptibility allele. Since the human and the murine class II alleles associated with IDDM share conserved features, cross-reactive T cell recognition of GAD65 and Cox P2-C may contribute to the pathogenesis of human IDDM and account for the epidemiological association of coxsackievirus with IDDM.

Naïve, potentially autoreactive T cells can persist indefinitely in healthy individuals, “ignorant” of the endogenous autoantigen. However, if these naïve T cells become activated and differentiate into effector/memory cells, autoimmune disease may result (1). Therefore, priming of autoantigen-specific T cells is critical for the development of autoimmunity and it is widely anticipated that microbial antigens which cross-react with host proteins are the etiologic agents that initiate an autoimmune T cell response (2).

Microbial antigens that are candidates for triggering cross-reactive T cell responses fall into two categories. The first consists of homologous proteins that are conserved through evolution and that are expressed by both the infectious agent and the host. Owing to their overall sequence homology, antigen processing and presentation of the two homologous proteins are likely to produce identical or closely related determinants. The best-studied example in this category is the cross-reactive T cell recognition of mycobacterial heat shock protein (HSP) and host HSP which can induce experimental autoimmune disease (for a review see reference 3).

The second category of antigens that are candidates for

triggering cross-reactive T cell responses are microbial proteins that share limited regions of amino acid sequence similarity with a nonhomologous host protein. The sequence similarity between the 40-kD coxsackievirus P2-C protein (Cox P2-C), a protein which is involved in viral replication, and the 65-kD glutamate decarboxylase (GAD65), a neurotransmitter synthesizing enzyme expressed in the brain and the β cells of the pancreas, is prototypic of this category (4). Both of these antigens have been associated with the pathogenesis of insulin-dependent diabetes mellitus (IDDM). Coxsackievirus has been experimentally and epidemiologically associated with IDDM (4–10) and T cell autoimmunity to GAD65 has been shown to play a key role in the induction and propagation of β cell autoimmunity in murine IDDM (11, 12) and may also play a role in human IDDM (13). These two antigens share a stretch of six consecutive amino acids in otherwise unrelated primary amino acid sequences.

Although small regions of sequence similarity (usually spanning four to six amino acids) are frequently identified by computer searches of DNA and protein databases, it remains unclear whether they actually can be cross-reactively recognized

by T cells after the natural processing of the whole antigens. Experimental evidence in support of T cell cross-reactivity between sequence similarities in nonhomologous proteins of microbial and host origin is limited to two examples: (a) T cells primed by a synthetic peptide of hepatitis B virus polymerase which shares 6 of 10 amino acids with myelin basic protein can induce experimental allergic encephalomyelitis (14). However, since the use of a synthetic peptide bypasses the antigen-processing compartment, it is unknown whether the cross-reaction also occurs after exposure to, and processing of, the native antigens. (b) Immunization with yeast histone, which contains a stretch of five amino acids that is identical to a region in retinal binding protein, can induce uveitis (15).

The rules for cross-reactive T cell recognition of sequence similarity regions in unrelated proteins have not been systematically studied. Based on our understanding of antigen processing and presentation, T cell cross-reactivity between sequence similarities in nonhomologous proteins should be a rare event that is dependent upon the nonoverlapping amino acid sequences of both antigens as well as the MHC haplotype of the host. The unrelated primary sequences of the two proteins should lead to unique patterns of cleavage sites for proteases. Therefore, during the natural processing of the whole antigens, peptides containing the sequence similarities may, or may not, be generated from either antigen for binding to MHC (for a review see reference 16). If generated, both peptides encompassing the sequence similarity region have to display high affinity for the same MHC product, high enough to constitute a dominant determinant (usually, only one peptide from a complex protein constitutes a dominant determinant). Whereas endogenously generated determinants are 10–15 amino acids long, the regions of sequence similarity are considerably shorter (4–6 amino acids). Consequently, differences in residues flanking the sequence similarity may profoundly affect each peptide's affinity for the MHC molecule, the MHC anchor residues employed, and the spatial orientation of the sequence similarity residues on the MHC molecule, such that cross-reactivity fails to occur.

To be recognized by a single TCR when bound to MHC molecules, both peptides have to display amino acids of the sequence similarity region in identical spatial orientation relative to the MHC molecule itself. Therefore, based on the nature of antigen recognition by T cells, cross-reactive recognition of sequence similarity regions on unrelated proteins should be a rare, MHC allele-dependent event. However, this prediction, which may be fundamental to the understanding of autoimmune disease, has not been experimentally addressed.

Choosing the coxsackievirus/GAD65 paradigm, we tested whether cross-reactive T cell recognition of the sequence similarity regions on these two otherwise unrelated proteins would occur, and if it occurred, how frequent permissive MHC alleles are. We report that of 10 murine restriction elements tested, cross-reactive presentation occurred in the context of only one, the "diabetes susceptibility allele," I-A^{Nod}.

Materials and Methods

Mice. Nonobese diabetic (NOD mice) (Taconic Farms Inc., Germantown, NY) were maintained and bred under specific pathogen-

free conditions. Bm12, SJL/L, B10.Q, B10.A (4R), C57.BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.GD mice were provided by Eli Sercarz (University of California, Los Angeles).

Antigens. Recombinant human GAD65, Cox P2-C, and *Escherichia coli* β -galactosidase were purified from recombinant *E. coli* on the basis of a histidine tag and metal affinity chromatography (11, 17). Peptides were synthesized using standard Fmoc chemistry and purified by chromatography. The peptide composition was verified by mass spectrometry. The GAD65 sequence similarity peptide (GAD ssp) is: ARYKMFPEVKEKGMMAA. The Cox P2-C sequence similarity peptide (Cox ssp) is: LKVKILPEVKEKHEFL (see Fig. 1). The control hen eggwhite lysozyme peptide (HEL_{11–25}, AMKRHGLDNYRGYSL), immunogenic in NOD mice, was provided by Eli Sercarz.

Immunization Protocols. Male mice were immunized subcutaneously at the ages indicated with 7 nmol protein (Cox P2-C, GAD65, or β -galactosidase), or peptide (Cox ssp, GAD ssp, or HEL_{11–25}), emulsified in CFA (Difco Laboratories Inc., Detroit, MI). 9 d later, draining lymph nodes were assayed for proliferative T cell responses.

T Cell Proliferation Assays. Single cell suspensions of draining lymph node cells were cultured at 4×10^5 cells/well in triplicates in 96-well microtiter plates with HL-1 serum-free medium (Ventrax Laboratories, Portland, ME), 2 mM glutamine, 100 U penicillin, and 100 μ g streptomycin per ml. Antigens were present at 20 μ g/ml for whole proteins and 7 μ M for peptides (the optimal concentrations determined in our previous study; 11). Con A (2.5 μ g/ml) or purified protein derivative of mycobacterium tuberculosis (PPD) (5 μ g/ml) was used as a positive control. [³H]thymidine was added during the last 12–18 h of a 5-d culture and incorporation of label was measured by liquid scintillation counting.

Results and Discussion

GAD65 and Cox P-2C ssp Induce Cross-reactive T Cell Responses only in NOD Mice. We synthesized the peptides that encompass the sequence similarity region in Cox P2-C and GAD65 (Cox ssp and GAD ssp, Fig. 1) and tested whether these peptides are immunogenic in the context of a panel of H-2 class II alleles (Table 1). First, we focused on various I-E⁻ mouse strains, which permitted us to test for I-A-restricted responses. Subsequently, if a particular I-A haplotype did not restrict a T cell response, we included mouse strains that also expressed I-E molecules to also include these possible restriction elements in the screen. Out of the seven I-A⁺, I-E⁻ mouse strains tested, which encompassed a spectrum of standard I-A alleles, GAD ssp and Cox ssp were immunogenic only in NOD mice (Table 1, columns A and B). We then tested three strains which, in addition, expressed the I-E alleles d, k, and u, respectively, but these I-E alleles did not restrict a T cell response to the peptides. Thus, out of 10 potential mouse class II restriction elements, only one, I-A^{Nod}, restricted a T cell response to GAD ssp and Cox ssp.

These data show that GAD ssp and Cox ssp can both bind

GAD65 255 A R Y K M F P E V K E K G M A A 269
Cox. P2-C 32 L K V K I L P E V K E K H E F L 47

Figure 1. The mouse GAD65 (27) and coxsackievirus sequence similarity peptides (GAD ssp and Cox ssp) (4). Identical amino acid residues are boxed. Numbers refer to the number of amino acid residues from the NH₂ terminus of each protein.

Table 1. *GAD65 and Cox P2-C Sequence Similarity Peptides (GAD ssp and Cox ssp) Induce Cross-reactive T Cell Responses, but only in the Context of a Diabetes-associated Class II MHC*

Strain	Ia allele expressed		Lymph node T cell proliferation (cpm $\times 10^3$)			
	I-A	I-E	A	B	C	D
			Immunized: Cox ssp Recall: Cox ssp	GAD ssp GAD ssp	Cox ssp GAD ssp	GAD ssp Cox ssp
Bm 12	6m12	—	1.9	2.0	2.1	2.9
SJL	s	—	2.4	2.5	2.4	2.7
B10.Q	q	—	2.3	2.4	2.5	2.8
B10.A(4R)	k	—	2.3	2.2	1.9	1.8
C57.BL/6	b	—	2.4	2.5	2.1	2.3
B10.GD	d	—	2.7	1.9	2.4	1.9
NOD	nod	—	65.3	67.8	32.5	46.6
A/J	k	k	2.1	2.2	2.3	2.5
B10.PL (73ns)	u	u	2.4	2.5	2.9	2.5
BALB/c	d	d	3.6	2.3	2.8	2.8

Lymph node T cell responses to GAD ssp and Cox ssp were tested in 10 strains of mice. Three to five male mice were immunized at 8 wk of age and tested 9 d later in two separate experiments. Background for medium alone ranged from 1,500 to 2,400 cpm. Antigen-induced proliferation that was >threefold over background is indicated in bold. As a positive control, PPD (5 μ g/ml) induced proliferation of about 38,000–64,000 cpm in all strains of mice. Data are expressed as average cpm $\times 10^3$. Cross-reactivity was also observed at lower antigen concentrations (ranging from 7 to 0.8 μ M) in NOD mice. There were no detectable spontaneous T cell responses to either peptides in the lymph node population of uninjected or β -Gal-immunized (Fig. 2) NOD mice.

to I-A^{nod}, but owing to their sequence dissimilarities, TCRs may discriminate between the two peptide-MHC complexes. However, we found that GAD ssp and Cox ssp are cross-reactively recognized: T cells primed by Cox ssp showed clear proliferative recall responses to GAD ssp and vice versa (Table 1, columns C and D). The response recalled by the unrelated ssp was comparable in magnitude to the one recalled by the cognate peptide, suggesting that most T cells in the lymph node population that recognized the nominal peptide also cross-reactively recognized the other ssp. Tolerance experiments also suggested that cross-reactivity was complete: tolerization of NOD mice to Cox ssp completely abrogated responsiveness to GAD ssp and vice versa (not shown). The data demonstrate that, in spite of the sequence dissimilarities in the flanking regions, both peptides bind to I-A^{nod} such that T cell cross-reactivity occurs.

It is possible that in the context of some of the H-2 haplotypes tested, the lack of immunogenicity by either sequence similarity peptide (Table 1) may not have resulted from a failure to bind to MHC, but rather from self-tolerance that had been established to the GAD65 sequence similarity determinant. In this case, complete cross-reactive presentation of both sequence similarity determinants had to occur, because self-tolerance to the GAD65 sequence similarity determinant completely abrogated responsiveness to Cox ssp.

Since the motifs of peptide binding differ among the various allelic products of the I-A and I-E locus, and the two ssp show considerable sequence variation (Fig. 1), we expected to observe four different restriction patterns in the panel of mouse strains tested: (a) neither ssp has affinity for a partic-

ular allelic product and neither primes a T cell response; (b) one ssp, but not the other, constitutes a determinant; (c) both ssp are immunogenic, but their spatial orientation in the MHC-peptide complex is unique for each peptide, thus preventing T cell cross-reactivity; and (d) both ssp are immunogenic and are cross-reactively recognized. It is surprising that of the 10 allelic class II products tested, we did not detect evidence for discordant binding (possibilities b and c). The pattern that we found may be biased due to conserved substitutions in the nonidentical residues between the two peptides. Most likely, the number of different class II products that we screened might not have been high enough to encompass all four binding patterns.

Cross-reactive priming occurred with I-A^{nod}, but not with I-A^d (Table 1), which differs in only two nonconserved amino acids from I-A^{nod} in the relevant peptide binding domain (18). Therefore, the two amino acid differences between the two alleles resulted in a unique phenotype. Even single amino acid substitutions on MHC molecules may have profound effects on peptide binding (19–21). Therefore, the serologically defined class II specificities of humans (e.g., DR3), which frequently encompass as many as 20 minor modifications in primary amino acid sequence, may display unique peptide binding properties depending on the specific expressed allele. Hence, only a certain subtype of a serologically defined specificity may be capable of restricting a cross-reactive T cell response and function as a “susceptibility allele” for the induction of autoimmune disease.

Whole GAD65 and Cox P2-C Induce Cross-reactive T Cell Responses. Whereas the above experiments demonstrate that

peptides that encompass the sequence similarity region can bind to I-A^{nod} and can be cross-reactively recognized by T cells, these determinants might not actually be generated as dominant determinants after the natural processing of the whole antigens. If GAD65 and Cox P2-C share a T cell determinant, immunization with one of these antigens should induce a T cell response that cross-reacts with the other antigen, and this response should be specific for the sequence similarity region. Groups of NOD mice were immunized with either the whole antigens (Cox P2-C, GAD65, and control β -galactosidase), or with the peptides (Cox ssp, GAD ssp, and control HEL₁₁₋₂₅). Subsequently, the recall response to all of these antigens was tested in each mouse.

The results showed that, after natural processing of both GAD65 and Cox P2-C, the sequence similarity regions were generated as dominant determinants: Cox ssp recalled a T cell response primed by whole Cox P2-C (Fig. 2 *c*) and vice versa (Fig. 2 *d*). Similarly, GAD ssp recalled proliferation in the T cell population primed with whole GAD65 (Fig. 2 *e*) and GAD ssp-primed cells proliferated to whole GAD65

and Cox P2-C (Fig. 2 *f*). Mice immunized with GAD65, Cox P2-C, and the sequence similarity peptides showed no responses to control β -galactosidase and HEL₁₁₋₂₅. Mice immunized with the control antigens displayed no lymph node T cell reactivity to GAD65 or Cox P2-C or their ssp (Fig. 2, *A* and *B*).

With most protein antigens studied so far, processing of the native antigen results in the presentation of only one, or a few peptide sequences as dominant determinants (16). It is intriguing that among the vast number of potential peptide fragments on the two proteins (GAD65 is 585 and Cox P2-C is 329 amino acids), both proteins were processed to produce the sequence similarity region as a dominant determinant. Since the two proteins are otherwise unrelated, the dominant display of the sequence similarity determinants occurred despite the existence of unique cleavage sites for lysosomal processing and in the presence of a number of other potential determinants on the molecules that might have had higher affinity for MHC binding.

Most importantly, cross-reactive recognition of the sequence

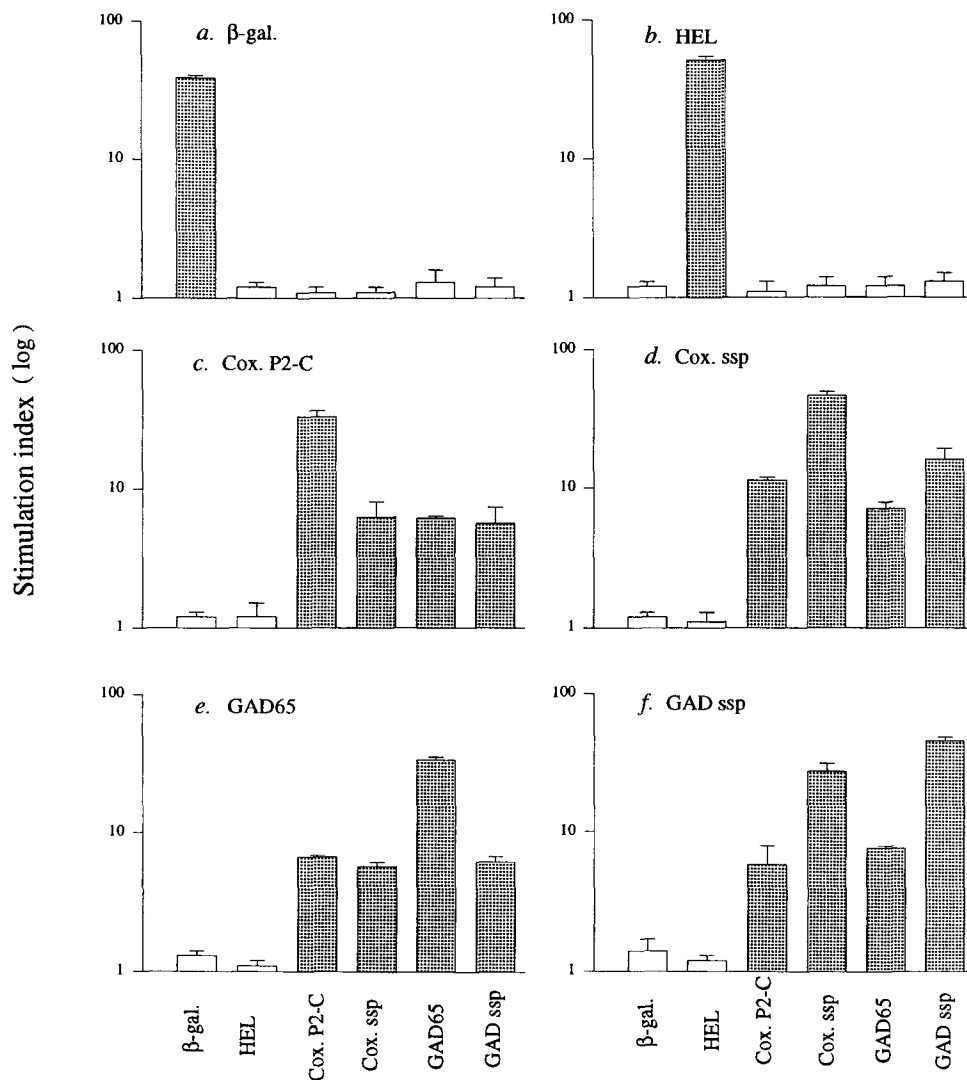


Figure 2. T cell cross-reactivity between GAD65 and Cox P2-C. NOD mice were immunized at 3 wk of age with control β -Gal (a), control HEL₁₁₋₂₅ peptide (b), whole Cox P2-C (c), Cox ssp (d), whole GAD65 (e), or GAD ssp (f). 9 d after immunization, lymph nodes were assayed for T cell responses to all six antigens. Three to four mice from each experimental and control group were assayed simultaneously in two separate experiments. The data from both experiments were pooled and the data are expressed as stimulation index (SI) \pm SEM. (Shaded bars) Antigens that triggered significant T cell responses (SI ≥ 3.0). The background in all experiments was ~ 500 – $2,000$ cpm.

similarity regions also occurred after natural processing of the whole Cox P2-C and GAD65. After priming with whole Cox P2-C protein, GAD65 and GAD ssp recalled a response of comparable magnitude to that of recall with the Cox ssp itself (Fig. 2 c). Also, in the T cell population primed with whole GAD65, responses to GAD ssp, Cox ssp, and whole Cox P2-C protein were recalled and were of comparable magnitude (Fig. 2 e). Apparently, the determinants generated by proteases during processing of the whole antigens yielded determinants that behaved in a manner identical to that of the ssp we synthesized (Fig. 1). Furthermore, these naturally generated determinants were displayed on I-A^{nod} molecules indistinguishably, with regard to T cell recognition.

In conclusion, we have demonstrated that cross-reactive T cell recognition of the Cox P2-C/GAD65 sequence similarity occurs in the context of only 1 out of 10 MHC alleles tested. The observed constraints on T cell cross-reactivity imposed by the processing and presentation of antigen may explain why common pathogens do not induce widespread T cell-mediated autoimmune diseases despite the frequent sequence similarities present in common pathogens. T cell cross-reactivity may only be inducible in a small fraction of a population that possesses specific MHC susceptibility alleles. It is striking that dominant display and cross-reactive recognition of the sequence similarity regions within the two diabetes-associated antigens occurred only in the context of a diabetogenic MHC allele. This suggests that the cross-reactivity we observed may not have been a random event but rather a result of molecular mimicry, i.e., the evolutionary adaptation of a dominant coxsackievirus determinant to mimic a dominant GAD65 determinant in order to evade a host immune response. Coxsackievirus has a broad host range, and mimicry may have evolved to MHC alleles that share peptide

binding motifs among these species. This adaptation may provide a selective advantage to the virus in hosts whose MHC alleles cross-reactively present the two determinants but fail to respond to the viral determinant due to self-tolerance to the GAD65 determinant. In contrast, this adaptation may result in autoimmunity in hosts that have not established self-tolerance to the GAD65 determinant. If we exclude the diabetogenic allele from consideration (because the observed cross-reactivity may be nonrandom), the frequency of MHC alleles capable of restricting a cross-reactive response may be far less than 1 in 10 within a broader spectrum of allelic restriction elements, as occurs in the human population.

Whereas coxsackievirus infection is not suspect in the pathogenesis of murine IDDM, it has been epidemiologically associated with human IDDM (8–10). The murine and human class II susceptibility alleles for IDDM share structural features (18, 22, 23). In addition, autoreactive T cells from NOD mice and IDDM patients appear to recognize similar determinants in GAD65 (11, 24, 25), and HSP (26). Therefore, the cross-reactive presentation of the Cox P2-C and GAD65 that we have observed in NOD mice may also occur with the context of human susceptibility alleles. Consistent with this hypothesis, peptides containing the GAD65 region of sequence similarity with coxsackievirus bind well to human DQw8, a MHC haplotype that confers susceptibility to IDDM (Wicker, L., personal communication) and are the major determinant recognized by PBMC from individuals at high risk for IDDM (24). Thus, the T cell cross-reactivity that we describe here in a murine model system may, in humans, contribute to the initiation or amplification of β cell autoimmunity and the association of IDDM with certain HLA alleles.

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